Regional Heterogeneity in Human Corneal and Limbal Epithelia: An Immunohistochemical Evaluation

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The authors studied the distribution of specific keratins within the superior, inferior, medial, and lateral regions of human limbus and cornea to determine whether the limbal epithelium exhibits regional heterogeneity in its microstructure. A corneal epithelial basic keratin (K3), recognized by monoclonal antibody AES, was immunohistochemically undetectable in the basal layers of the limbus in these four regions, but was seen in all layers in the central cornea. The pattern of immunostaining with another monoclonal antibody, AE1, which recognizes several acidic keratins, was complementary to AE5 staining in that AE1 recognized a similar heterogeneity in the limbal epithelial cells. AE1 immunoreacted with the basal cells of the limbus, but not those of the central corneal epithelium. Limbal characteristics, as defined by AE1-positive and AE5-negative staining, extended deeply into peripheral cornea in the superior and inferior regions, but to a lesser extent in the lateral and medial regions. The broader regions of epithelium with limbal characteristics in the superior and inferior regions raises the possibility that these regions play an important role in corneal epithelial maintenance and wound healing.

Several lines of evidence suggest that peripheral corneal epithelial cells migrate centripetally. Davenger and Evensen1 showed that in certain black patients, the heavily pigmented limbal cells form streaks toward the central cornea. Bron2 extended this observation and noted that in numerous corneal epithelial diseases, such pigmented or otherwise marked peripheral corneal epithelial cells form similar streaking patterns; this suggests centripetal migration. This concept received support from Aldredge and Krachmer,3 who found that the epithelium of transplanted donor human cornea was gradually replaced by peripheral cells. Kinoshita et al4 used the sex chromosome as a definitive marker of limbal cells to provide evidence that centripetal migration also occurs in experimentally transplanted rabbit cornea. Buck5 showed that peripheral rat corneal epithelial cells, labeled (tattooed) by carbon particles, migrate centripetally and showed that this cell movement occurs even in normal, minimally traumatized cornea. He also showed that hemidesmosomes are arranged in parallel arrays that point toward central cornea, and thus provide a possible mechanism for oriented cell sliding. Based on these data, Thoft and Friend6 proposed an “X,Y,Z hypothesis of corneal epithelial maintenance” in which the desquamated cells (Z component) are continuously replaced not only by the basal cells (X) that divide but also by cells that migrate in from the periphery (Y).

If cells migrate centripetally, what is the ultimate source of these cells? Davenger and Evensen1 and Bron2 provided theoretic arguments that implied that human limbal epithelium is associated with specialized rete ridges called palisades of Vogt. However, these data on cell migration cannot rule out the possibility that conjunctival epithelial cells can migrate over the limbus onto the cornea proper and transdifferentiate into corneal epithelium.

Schermer et al7 studied the expression of different keratin proteins in rabbit corneal epithelium. They and others8–14 had shown that there are a total of over 20 keratins and that different pairs of acidic and basic keratins are expressed in a tissue in differentiation-stage-specific manner. With cultured rabbit corneal epithelial colonies as a model, Schermer et al1 showed that a basic 64K keratin (K3) and an acidic 55K keratin (K12) are characteristic of suprabasal cell layers and suggested that these two keratins may be regarded as molecular markers for an advanced stage of corneal epithelial differentiation. When the monoclo-
nal antibody (AE5) against the 64K keratin was used to stain frozen corneal sections, they found that although K3 is suprabasally located (as expected) in limbal epithelium, this keratin can be detected in the entire thickness of central corneal epithelium (basal cells included). Weak, if any, AE5 staining can be detected in conjunctival epithelium. These results suggest that as far as keratin expression is concerned, limbal cells are intimately related to corneal epithelium and that central corneal epithelial basal cells are in a more differentiated state than limbal basal cells. These findings, in conjunction with the data that exist on centripetal cellular migration, have led to the proposal that corneal epithelial stem cells are located in the limbal basal layer.7

There are data that support this proposal that corneal epithelial stem cells are located in the limbal basal layer. Ebato et al13 showed that under explant culture conditions, human limbal epithelial cells grow much better than central corneal epithelial cells. Kenyon and Tseng16 reported that when limbal epithelium was included in conjunctival transplantation in ocular surface disorders, the chance of successful re-epithelialization was greater. A report by Kinoshita et al10 showed that rabbit corneal epithelium can be regenerated from limbal epithelium, and another report by Roper-Hall17 showed that an important prognostic factor in alkali burns is the extent of damage to limbus. In addition, Cotsarelis et al18 established the existence of a population of limbal basal cells that are normally slow to cycle, but can be preferentially stimulated to proliferate by a tumor promoter (TPA) or by the physical removal of central corneal epithelium. These limbal cells fulfill the kinetic criteria of stem cells. Because these cells that cycle slowly were found to be absent from the central corneal epithelium, the data strongly suggest that corneal epithelial stem cells are located preferentially, if not exclusively, in the limbus.18

The pigment migration pattern cited earlier provided indirect evidence that centripetal cell migration from different regions of the limbus may occur at different rates. To test the possibility that human limbal epithelium may show some regional heterogeneity, we prepared frozen sections of superior, inferior, lateral, and medial limbus and stained them with AE5 as well as AE1, an antibody that recognizes a subset of acidic keratins.19,20 Previous studies have established that both antibodies produce tissue- and cell layer-specific staining in corneal and limbal epithelia.7,21,22 Kolega et al23 reported that the heterogeneity in K3 expression in the limbus correlates with the heterogeneous distribution of a basement membrane component recognized by a monoclonal antibody, AE27. In this study, we observed that the limbal epithelial staining characteristics (basal cells were AE5-negative but AE1-positive) extend deeply into the cornea proper in the superior and inferior regions, but only marginally in the medial and lateral regions. However, the correlation between AE5 and AE27 staining did not extend beyond the beginning of Bowman’s membrane. The clinical and biological implication of this regional heterogeneity will be discussed.

Materials and Methods

Four pairs of human donor eyes (ages 72, 78, 83, and 83) obtained from the Medical Eye Bank of Western Pennsylvania (Pittsburgh, PA) were examined with the slit lamp to ensure that there was no ocular surface disease or previous surgery. The cornea and adjacent sclera were excised from the whole globe. From each donor, one cornea was cut horizontally to include lateral and medial regions in the tissue sections, and the other cornea was cut vertically to include superior and inferior regions. The corneal halves were then frozen in Tissue-Tek II OCT compound (Miles Laboratory, Inc., Elkhart, IN) and stored at −70°C.

Cryostat sections (7 μm) of frozen tissue were transferred onto gelatin-coated microscope slides and immunostained with monoclonal antibodies AE5, AE1, or AE27. Hybridoma culture supernatants that contained AE1 and AE5 (prepared as previously described7,19,20) or AE2723 antibodies were used for the immunostaining. Indirect immunochemical staining was performed as previously described.24 To reduce nonspecific binding of fluorescein isothiocyanate-labeled rabbit anti-mouse IgG (Organon-Technika, West Chester, PA), the labeled antibody was absorbed at 4°C for 48 hr with the nonsoluble fraction of two homogenized human corneas. After absorption, the supernatant with fluorescein-conjugated rabbit anti-mouse IgG was diluted 1:40 with 10% heat-inactivated rabbit serum in phosphate-buffered saline and used for immunostaining.

To study topographic differences in the keratin distribution in the peripheral cornea and the limbus, the boundary between the limbus and corneal epithelium was identified by two criteria. The first criterion was the location of the beginning point of the underlying Bowman’s membrane. The second was the location of the transition of blood vessels in the underlying stroma. Blood vessels were identified by immunostaining their basement membranes with anti-laminin antibodies. In several sections (that included superior, inferior, lateral, or medial regions of the cornea and limbus), the blood vessels in the underlying stroma terminated at the location of the beginning of Bowman’s membrane. Therefore, the be-
beginning of Bowman's membrane was used as the reference point to analyze the regional distribution of keratins in the limbus and the cornea. The slides were examined by an Olympus Photomicroscope (Tokyo, Japan) with epifluorescence and phase contrast objectives. An image processor was used to measure the linear distance from the origin of the Bowman's membrane to specific regions of the limbus or cornea. To determine whether the regional differences were statistically significant, statistical analyses were performed with the ANOVA test. Because the values were not normally distributed within each group (superior, inferior, lateral, or medial), Bonferroni t-test for multiple comparisons was also performed with log-transformed values.

Results

Cross-sections of the tissues that included the lateral and medial limbus and peripheral cornea gave this pattern of immunostaining keratins recognized by AE5 and AE1 monoclonal antibodies; In the limbus, AE5 immunostained the superficial epithelial cell layers. The staining extended to deeper cell layers toward the origin of Bowman's membrane (Fig. 1). The basal layers of the epithelium in the limbus were not stained with AE5 in any of the specimens. In the peripheral cornea (Fig. 1), AE5 staining was less intense in the basal epithelium, but toward the central cornea, all the layers stained uniformly and intensely (Fig. 2).

Alternate serial sections of the tissues were stained with AE1. The distribution of keratins, recognized by AE1, was inversely related to the distribution of the 64K keratin (K3) recognized by AE5. Limbal basal cells immunostained brightly with AE1 (Fig. 3), whereas the cells above the basal layer reacted weakly and heterogeneously. The staining ended in regions near the origin of Bowman's membrane. In the central cornea (Fig. 4), only the superficial layer of epithelium was immunostained with AE1.

Immunostaining of the tissue, sectioned vertically through the cornea to include the superior and inferior regions of the limbus, gave these results; in the limbus, AE5 labeled 5 to 10 aggregates of cells per section of tissue. The absence of staining in the basal layer extended into the cornea, beyond the origin of Bowman's membrane (Fig. 5). AE1 staining in the superior and inferior limbal and corneal regions is shown in Figure 6. Cells in the basal layers in limbus stained strongly with AE1, and this staining pattern extended to peripheral cornea, beyond the origin of Bowman's membrane. The measurements of the widths of regions of the basal epithelium in the peripheral cornea, devoid of AE5 staining, are seen in
Fig. 3. Immunofluorescence staining of a corneal section containing lateral limbus and peripheral corneal epithelium. Top, micrograph of the section reacted with monoclonal antibody AE1; bottom, corresponding phase contrast image. The origin of Bowman’s membrane is indicated by an arrow. Suprabasal layers of the epithelia in the limbal region stain moderately and heterogeneously with AE1, whereas the basal cells stain intensely. The basal cell subpopulation which reacts with AE1 extends only up to a region close to but not past the origin of Bowman’s membrane. Bar = 50 μm.

Figure 7. In these measurements, the origin of Bowman’s membrane was used to define the boundary between the cornea and the limbus. Similarly, measurements of the peripheral corneal regions that show the presence of AE1 staining in the basal epithelium are seen in Figure 8. In a separate study on the regional comparison of the mitotic index, basal cells per unit length were counted. Based on these counts, no significant regional differences were noted in the cell density. Therefore, the lengths measured in Figures 7 and 8 are directly related to the cell number. The statistical analyses indicated that the superior corneal region that showed limbal staining characteristics was significantly larger than the inferior, lateral, or medial region; similarly, the inferior region was larger than the lateral or medial region. However, the lateral and medial regions were not significantly different from each other.

To study the relationship between K3 expression and the distribution of a basement membrane-associated antigen, recognized by AE27, serial sections of cornea were reacted with AE5 and AE27 and were compared (Figs. 9A and B, respectively). As described earlier, the basal cells in the limbus as well as the basal cells in the peripheral cornea in the superior and inferior regions did not show detectable levels of staining with AE5. However, the suprabasal layers of the epithelium in these regions showed a heterogeneous pattern of AE5-positive staining. Also, a heterogeneous pattern of staining (strong and weak staining regions) with AE27 was evident in the limbus. The regions that stained strongly with AE27 tended to coincide with overlying AE5-positive suprabasal regions. However, in the cornea, starting at the origin of Bowman’s membrane, the staining with AE27 appeared...
Vig%. immunofluorescence staining of a corneal section containing superior limbal and peripheral corneal epithelium. Top, a micrograph of a section reacted with monoclonal antibody AE5; bottom, corresponding phase contrast image. The origin of Bowman's membrane is indicated by an arrow. Intermittent staining is seen in the limbal epithelium. This pattern extends onto the adjacent peripheral cornea. Bar = 50 μm.

to be strong and continuous, with no correlation to AE5 staining in the overlying epithelium.

Discussion

The main conclusion of this study is that there is significant regional heterogeneity in the limbus and peripheral cornea. This heterogeneity is best visualized immunohistochemically with antibodies to keratins, a class of differentiation-dependent cytoskeletal proteins. AE5 antibody stained limbal epithelium suprabasally but central corneal epithelium uniformly. This finding suggests that the 64K keratin (K3) expression is regulated differently in these two regions. The cellular and molecular mechanism for this differential expression is unknown, but could be related to different basement membrane compositions or different microenvironments provided by the subepithelial tissues. Type IV collagen is readily detectable immunohistochemically in the basement membrane of conjunctival and limbal epithelium, but not in the corneal basement membrane overlying Bowman's membrane. Such a heterogeneity in Type IV collagen does not correlate well with the changes seen in (basal vs suprabasal) AE5 staining patterns. On the other hand, another type of basement membrane heterogeneity, as defined by a new monoclonal antibody, AE27, showed a correlation between the AE27 positive basement membrane regions and K3 expression by the basal cells.23 Only the basal cells that rested on the AE27-positive regions tended to express K3. In central cornea, the basement membrane was AE27-positive and the basal cells that rested on this basement membrane were AE5 positive. However, this relationship between the absence of K3 and weak or no staining of underlying basement membrane with AE27 was not shown beyond the origin of Bowman's membrane in the cornea. In the superior and inferior peripheral corneal regions, although there were AE5-negative basal epithelial cells, AE27 staining was...
Fig. 7. Bar graph showing the measurements of regions of peripheral corneal epithelium from the beginning of the Bowman’s membrane to a point where all the epithelial layers begin to react with monoclonal antibody AE5. Each bar represents the average measurements (±SEM) from at least four sections taken 50 μm apart. The four corneas are from different human donors.

strong and continuous. The reason for this difference between the peripheral cornea and the limbus, with respect to the relationship of K3 with the basement membrane heterogeneity, may be that there is more than one mechanism that controls K3 expression. The mechanisms of the control of K3 expression in the limbal stem cells may be distinctly different than the population of differentiated epithelial cells that have a high proliferative potential. AE5-negative basal cells in the peripheral cornea would, thus, belong to the latter category. The heterogeneity in the limbal basement membrane may be due to localized alterations in basement membrane components that may influence stem cells to attach to or detach from their underlying basement membrane.

Another mechanism used to obtain different (basal vs suprabasal) AE5 staining patterns in corneal epithelium relates to cell proliferation. We and others have shown that the regenerated corneal epithelium, even when it is associated with apparently normal corneal basement membrane, is stained suprabasally by AE5. However, the staining returns to a normal, uniform pattern once the wound is healed. Similar results were obtained when AE5-positive rabbit corneal epithelial basal cells were plated in culture. Some of these cells would attach and proliferate and, thus, lose their AE5 staining within 2–3 days. Pre-

sumably, the loss of AE5 staining in these activated basal cells is due to the termination of the syntheses of the 64K (K3) and 55K (K12)-differentiation-related keratins that are diluted out when cell proliferation occurs. Later, when the cells reach confluency and become heavily stratified, they turn on the syntheses of K3/K12 that first appear only suprabasally. K3 appears in the basal cells only later when the culture becomes senescent. Although one cannot rule out the possibility that subtle changes in the basement membrane or extracellular matrix may be involved in the wounding/cell culture conditions, the data suggest that cell proliferation can have a major effect on K3 expression.

We do not know whether these mechanisms (basement membrane heterogeneity, differences in the subepithelial tissues or cell proliferation) may play a role in the limbal heterogeneity. It is relevant to note, however, that the proliferative rate of the limbal cells is actually roughly equal to that of central corneal epithelium, and that the limbal proliferative rate does not seem to show any regional heterogeneity. This conclusion is based on several lines of investigation. Kaufman measured the mitotic index (MI) of rat limbal, peripheral, and central corneal epithelia, and found no significant difference in the MI of central vs limbal epithelia. He further noted that superior, infe-
Fig. 9. Immunofluorescence staining of corneal section containing superior limbal and peripheral corneal epithelium. (A) Micrograph on the top, tissue section was reacted with monoclonal antibody AE5, and bottom, corresponding phase contrast photomicrograph. (B) Top, serial section of the tissue reacted with antibody AE27 and bottom, corresponding phase contrast photomicrograph. The origin of Bowman's membrane is indicated by an arrow. Note a continuous pattern of AE27 staining which starts in a close proximity of the beginning of Bowman's membrane and extends to the central cornea. Bar = 75 μm.

rior, lateral, and medial limbus have about the same MI. Similar conclusions were drawn by Haskjold et al.,27,28 who measured colcemid-arrested mitoses in rat corneal/limbal epithelia. Unpublished data on human and mouse limbal epithelia also support these notions (Robert Lavker, personal communication, 1990). Therefore, it seems clear that the number of cells per unit length of tissue that divided is roughly the same along the entire circumference of the limbus. If so, the limbal heterogeneity described here, as shown by anti-keratin staining, may have nothing to do with hot spot(s) of cell proliferation. We cannot rule out the possibility, however, that AE5-negative basal cells may have a higher proliferative potential. Slow centripetal migration from the limbus may provide a source of cells with high proliferative potential.

In fact, data that exist from explant culture and wounding experiments seem to support this possibility. A corollary of this concept is that since superior and inferior limbus seem to possess a larger number of AE5-negative basal (stem) cells than lateral and medial limbus, the former zones may play a more important role than the latter in the long-term maintenance and regeneration of central corneal epithelium. More data are required to test this hypothesis.

The staining pattern produced by AE1 antibody is somewhat complementary to that produced by AE5. Thus, in the limbal region, beginning near the origin of Bowman's membrane, the basal cells tend to be AE1 positive but AE5 negative, whereas corneal basal cells are AE1 negative but AE5 positive. Since AE1 recognizes several acidic keratins and since the de-
tailed keratin composition of human limbal epithelium is unknown, the molecular basis of AE1 staining of limbal basal cells is unclear. Previous tissue surveys have established, however, that basal cells of all stratified squamous epithelia contain an acidic 50K (K14) keratin that, together with its partner, basic 58K (K5) keratin, represent the major keratins of basal cells.19,20,29–35 This may explain why AE1 stains basal cells of most stratified epithelia—this includes epidermis, esophageal epithelium, and epithelium in the limbal region.11,19,20 The absence of AE1 staining in central corneal epithelium is not surprising since this epithelium contains only one major acidic keratin (55K, or K12) that is not recognized by AE1. Central corneal epithelium contains little, if any, K14 that is characteristic of the basal cells of all other stratified epithelia. This finding is highly significant in that it highlights the uniqueness of central corneal epithelium, a lack of K14 keratin-containing basal cells. The fact that these K14-negative basal cells are actually engaged in the synthesis of large amounts of K3 keratin, the equivalents of which are usually associated with the suprabasal compartments in other more conventional stratified epithelia, again emphasizes the uniqueness of corneal epithelium. These considerations support our earlier suggestion that as far as keratin expression is concerned, the entire central corneal epithelial tissue is equivalent to the suprabasal compartment of other stratified epithelium.7

Conventionally, limbal epithelium is defined to be a narrow zone between the edge of Bowman’s membrane, marks the beginning of cornea, and a hypothetical line 2–3 mm lateral to the edge of Bowman’s membrane. In pigmented animals, this epithelium tends to be heavily pigmented, presumably designed for photoprotection. The morphologic transition between limbal and conjunctival epithelium is not always clear in humans whose conjunctival epithelium is, like limbal epithelium, highly stratified. However, bulbar conjunctival epithelium a few millimeters away from the limbus, as marked by mature goblet cells, stains weakly, if at all, with AE5 antibody and, thus, can be distinguished from limbal epithelium. The immunohistochemical definition of limbal epithelium has its limitation, however, in that superior limbal epithelium shows only weak or scattered AE5 staining. For this reason, we prefer the conventional definition of limbus based on the position of Bowman’s membrane, even though our data have established that this definition does not correlate with certain immunohistochemical aspects of regional differences.

Davenger and Evensen1 noted that in the healthy eyes of some African and Asian populations, some limbal cells are highly pigmented and form streaks in cornea. They pointed out that these streaks, best visible from inferior limbus, curve horizontally and come together at the position of the Hudson–Stahl line, that represents a border line of cell migration from inferior limbus. In another study, Bron2 described the pigment patterns in toxic keratopathies, Fabry’s disease, striate melanokeratosis, and iron deposition. Based on the fact that the Hudson–Stahl line is usually horizontal, Bron suggested that the rate of centripetal sliding was faster in the vertical than in the horizontal meridia. Because this line is usually located below the cornea center, it indicated that cell sliding was fastest from above. Although our data did not yield any information regarding cell migration, we do find more corneal invasion by cells with limbal characteristics in the superior limbus. We reported that in a human eye, a region of approximately 2 mm, which encompassed the limbus, was devoid of AE5 staining.36 Based on the current findings, we believe that the tissue analyzed in the previous study36 included the superior limbal region. Together, these data suggest the possibility that superior limbus, which contains more and better developed palisades of Vogt, may be endowed with more stem cells that yield progeny cells that undergo centripetal migration. This hypothesis has important clinical implications because it suggests that diseases or injury in these regions would be particularly harmful. By the same token, injury to the lateral and medial limbus, which contain smaller reserves of stem cells, is more likely to result in conjunctival ingrowth. The current findings suggest that the superior region, which includes the limbus and peripheral cornea, has a larger pool of epithelial cells with stem cell-like characteristics (absence of K3) and that these cells, therefore, have a higher proliferative potential. Thus, the superior region of the cornea and limbus may be an important source of cells that proliferate in response to epithelium injury. Also, according to this hypothesis, efforts to resurface the cornea via keratopitheliotomy, lenticule, or limbal transplants might be optimized with tissues from the superior or inferior limbus. The apparent lack of a stem cell population in the central human cornea may help explain why the epithelium does not renew in penetrating keratoplasty in patients with severe limbal diseases. Experiments are in progress to determine how the structure of the human limbus is altered in diseases such as superior limbic keratoconjunctivitis and contact lens-induced keratopathy.

Key words: cornea, limbus, keratins, corneal epithelium, limbal epithelium
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References